Fusion proteins for prodrug activation

The invention relates to compounds which contain an antigen binding region which is bound to at least one enzyme which is able to metabolize a compound (prodrug) which has little or no cytotoxicity to a cytotoxic compound (drug), where the antigen binding region is composed of a single polypeptide chain. It is advantageous for covalently bonded carbohydrates to be present on the polypeptide chain.

The combination of prodrug and antibody-enzyme conjugates for use as therapeutic composition has already been described in the specialist literature. This entails antibodies which are directed against a particular tissue and to which a prodrug-cleaving enzyme is bound being injected into an organism, and subsequently a prodrug compound which can be activated by the enzyme being administered. The action of the antibody-enzyme conjugate bound to the target tissue is intended to convert the prodrug compound into a compound which exerts a cytotoxic effect on the bound tissue. However, studies on antibody-enzyme conjugates have shown that these chemical conjugates have unfavorable pharmacokinetics so that there is only inadequate site-specific tumor-selective cleavage of the prodrug. Some authors have attempted to remedy this evident deficiency by additional injection of an anti-enzyme antibody which is intended to bring about rapid elimination of the antibody-enzyme conjugate from the plasma (Sharma et al.,

Brit. J. Cancer, 61, 659, 1990). Another problem of antibody-enzyme conjugates is the limited possibility of preparing large amounts reproducibly and homogeneously.

The object of the present invention was now to find fusion proteins which can be prepared on an industrial scale and are suitable, by reason of their pharmacokinetic and pharmacodynamic properties, for therapeutic uses.

It has been found in this connection that compounds which contain an antigen binding region which is composed of a single polypeptide chain have unexpected advantages for the preparation and use of fusion proteins, to which carbohydrates are advantageously attached, in prodrug activation.

The invention therefore relates to compounds which contain an antigen binding region which is bound to at least one enzyme, where the antigen binding region is composed of a single polypeptide chain, and carbohydrates are advantageously attached to the fusion protein.

An antigen binding region means for the purpose of the invention a region which contains at least two variable domains of an antibody, preferably one variable domain of a heavy antibody chain and one variable domain of a light antibody chain (sFv fragment). The antigen binding region can, however, also have a bi- or multivalent structure, i.e. two or more binding regions, as described, for example, in EP-A-0 404 097. However, a human or humanized sFv fragment is particularly preferred, especially a humanized sFv fragment.

The antigen binding region preferably binds to a tumor-associated antigen (TAA), with the following TAAs being particularly preferred:
neural cell adhesion molecule (N-CAM),
polymorphic epithelial mucin (PEM),
epidermal growth factor receptor (EGF-R),
Thomsen Friedenreich antigen β (TFβ),
gastrointestinal tract carcinoma antigen (GICA),
ganglioside GD_3 ($GD3$),
ganglioside GD_2 (GD_2),
Sialyl-Le^a, Sialyl-Le^x,
TAG72,
the 24-25 kDa glycoprotein defined by MAb L6,
CA 125 and, especially,
carcinoembryonic antigen (CEA).

Preferred enzymes are those enzymes which are able to metabolize a compound of little or no cytotoxicity to a cytotoxic compound. Examples are β-lactamase, pyroglutamate aminopeptidase, galactosidase or D-aminopeptidase as described, for example, in EP-A2-0 382 411 or EP-A2-0 392 745, an oxidase such as, for example, ethanol oxidase, galactose oxidase, D-amino-acid oxidase or α-glyceryl-phosphate oxidase as described, for example, in WO 91/00108, peroxidase as disclosed, for example, in EP-A2-0 361 908, a phosphatase as described, for example, in EP-A1-0 302 473, a hydroxynitrilelyase or glucosidase as disclosed, for example, in WO 91/11201, a carboxypeptidase such as, for example, carboxypeptidase G2 (WO 88/07378), an amidase such as, for example, penicillin 5-amidase (Kerr, D.E. et al. Cancer Immunol. Immunther. 1990, 31) and a protease, esterase or glycosidase such as the already mentioned galactosidase, glucosidase or a glucuronidase as described, for example, in WO 91/08770.

A ß-glucuronidase is preferred, preferably from Kobayasia nipponica or Secale cereale, and more preferably from E. coli or a human ß-glucuronidase. The substrates for the individual enzymes are also indicated in the said patents and are intended also to form part of the disclosure content of the present application. Preferred substrates of ßglucuronidase are N-(D-glycopyranosyl)benzyloxycarbonylanthracyclines and, in particular, N-(4-hydroxy3-nitrobenzyloxycarbonyl)doxorubicin and daunorubicin ß-D-glucuronide (J.C. Florent et al. (1992) Int. Carbohydr. Symp. Paris, A262, 297 or S. Andrianomenjanahary et al. (1992) Int. Carbohydr. Symp. Paris, A 264, 299).

The invention further relates to nucleic acids which code for the compounds according to the invention. Particularly preferred is a nucleic acid, as well as its variants and mutants, which codes for a humanized sFv fragment against CEA (carcinoembryonic antigen) linked to a human ß-glucuronidase, preferably with the ~~sequence indicated in Table 1~~ (sFv-huß-Gluc).

The compounds according to the invention are prepared in general by methods of genetic manipulation which are generally known to the skilled worker, it being possible for the antigen binding region to be linked to one or more enzymes either directly or via a linker, preferably a peptide linker. The peptide linker which can be used is, for example, a hinge region of an antibody or a hinge-like amino-acid sequence. In this case, the enzyme is preferably linked with the N terminus to the antigen binding region directly or via a peptide linker. The enzyme or enzymes can, however, also be linked to the antigen binding region chemically as described, for example, in WO 91/00108.

The nucleic acid coding for the amino-acid sequence of the compounds according to the invention is generally cloned in an expression vector, introduced into prokaryotic or eukaryotic host cells such as, for example, BHK, CHO, COS, HeLa, insect, tobacco plant, yeast or E.coli cells and expressed. The compound prepared in this way can subsequently be isolated and used as diagnostic aid or therapeutic agent. Another generally known method for the preparation of the compound according to the invention is the expression of the nucleic acids which code therefor in transgenic mammals with the exception of humans, preferably in a transgenic goat.

BHK cells transfected with the nucleic acids according to the invention express a fusion protein (sFv-huβ-Gluc) which not only was specific for CEA but also had full β-glucuronidase activity (see Example 5).

This fusion protein was purified by anti-idiotype affinity chromatography in accordance with the method described in EP 0 501 215 A2 (Example M). The fusion protein purified in this way gives a molecular weight of 100 kDA in the SDS PAGE under reducing conditions, while molecules of 100 and 200 kDa respectively appear under non-reducing conditions.

Gel chromatography under native conditions (TSK-3000 gel chromatography) showed one protein peak (Example 6, Fig. I) which correlates with the activity peak in the specificity enzyme activity test (EP 0 501 215 A2). The position of the peak by comparison with standard molecular weight markers indicates a molecular weight of $\approx$ 200 kDa. This finding, together with the data from the SDS PAGE, suggests that the functional enzymatically active sFv-huβ-Gluc fusion protein is in the form of a "bivalent molecule", i.e. with 2 binding regions and 2

enzyme molecules. Experiments not described here indicate that the fusion protein may, under certain cultivation conditions, be in the form of a tetramer with 4 binding regions and 4 enzyme molecules. After the sFv-huβ-Gluc fusion protein had been purified and undergone functional characterization in vitro, the pharmacokinetics and the tumor localization of the fusion protein were determined in nude mice provided with human gastric carcinomas. The amounts of functionally active fusion protein were determined in the organs and in the tumor at various times after appropriate workup of the organs (Example 7) and by immunological determination (triple determinant test, Example 8). The results of a representative experiment are compiled in Table 4.

Astonishingly, a tumor/plasma ratio of 5/1 is reached after only 48 hours. At later times, this ratio becomes even more favorable and reaches values > 200/1 (day 5). The reason for this favorable pharmacokinetic behavior of the sFv-huβ-Gluc fusion protein is that fusion protein not bound to the tumor is removed from the plasma and the normal tissues by internalization mainly by receptors for mannose 6-phosphate and galactose. (Evidence for this statement is that there is an intracellular increase in the β-glucuronidase level, for example in the liver).

As shown in Table 2, the sFv-huβ-Gluc contains relatively large amounts of galactose and, especially, mannose, which are mainly responsible for the binding to the particular receptors. The fusion protein/receptor complex which results and in which there is binding via the carbohydrate residues of the fusion protein is then removed from the extracellular compartment by internalization.

This rapid internalization mechanism, which is mainly mediated by galactose and mannose, is closely involved in the advantageous pharmacokinetics of the fusion protein according to the invention. These advantageous pharmacokinetics of the fusion protein to which galactose and, in particular, mannose are attached makes it possible for a hydrophilic prodrug which undergoes extracellular distribution to be administered i.v. at a relatively early time without eliciting non-specific prodrug activation. In this case an elimination step as described by Sharma et al. (Brit. J. Cancer, 61, 659, 1990) is unnecessary. Based on the data in Table 4, injection of a suitable prodrug (S. Adrianomenjanahari et al. 1992, Int. Carbohydrate Symp., Parts A264, 299) is possible even 3 days after injection of the sFv-huβ-Gluc fusion protein without producing significant side effects (data not shown).

A similarly advantageous attachment of carbohydrates to fusion proteins can also be achieved, for example, by secretory expression of the sFv-huβ-Gluc fusion protein in particular yeast strains such as Saccharomyces cerevisiae or Hansenula polymorpha. These organisms are capable of very effective mannosylation of fusion proteins which have appropriate N-glycosylation sites (Goochee et al., Biotechnology, 9, 1347-1354, 1991). Such fusion proteins which have undergone secretory expression in yeast cells show a high degree of mannosylation and favorable pharmacokinetics comparable to those of the sFv-huβ-Gluc fusion protein expressed in BHK cells (data not shown). In this case, the absence of galactose is compensated by the even higher degree of mannosylation of the fusion protein (Table 3). The sFv-huβ-Gluc fusion protein described above was constructed by genetic manipulation and expressed in yeast as described in detail in Example 9.

Instead of human β-glucuronidase it is, however, also possible to employ another glucuronidase with advantageous properties. For example, the E.coli β-glucuronidase has the particular advantage that its catalytic activity at pH 7.4 is significantly higher than that of human β-glucuronidase. In Example 10, an sFv-E.coli β-Gluc construct was prepared by methods of genetic manipulation and underwent secretory expression as functionally active mannosylated fusion protein in Saccharomyces cerevisiae. The pharmacokinetic data are comparable to those of the sFv-huβ-Gluc molecule which was expressed in yeast or in BHK cells (Table 4).

The glucuronidases from the fungus Kobayasia nipponica and from the plants Secale cereale have the advantage, for example, that they are also active as monomers. In Example 11, methods of genetic manipulation were used to prepare a construct which, after expression in Saccharomyces cerevisiae, excretes an sFv-B. cereus β-lactamase II fusion protein preferentially in mannosylated form.

This fusion protein likewise has, as the fusion proteins according to the invention, on the basis of β-glucuronidase pharmacokinetics which are favorable for prodrug activation (Table 4).

Furthermore, the compounds according to the invention can be employed not only in combination with a prodrug but also in the framework of conventional chemotherapy in which cytostatics which are metabolized as glucuronides and thus inactivated can be converted back into their toxic form by the administered compounds.

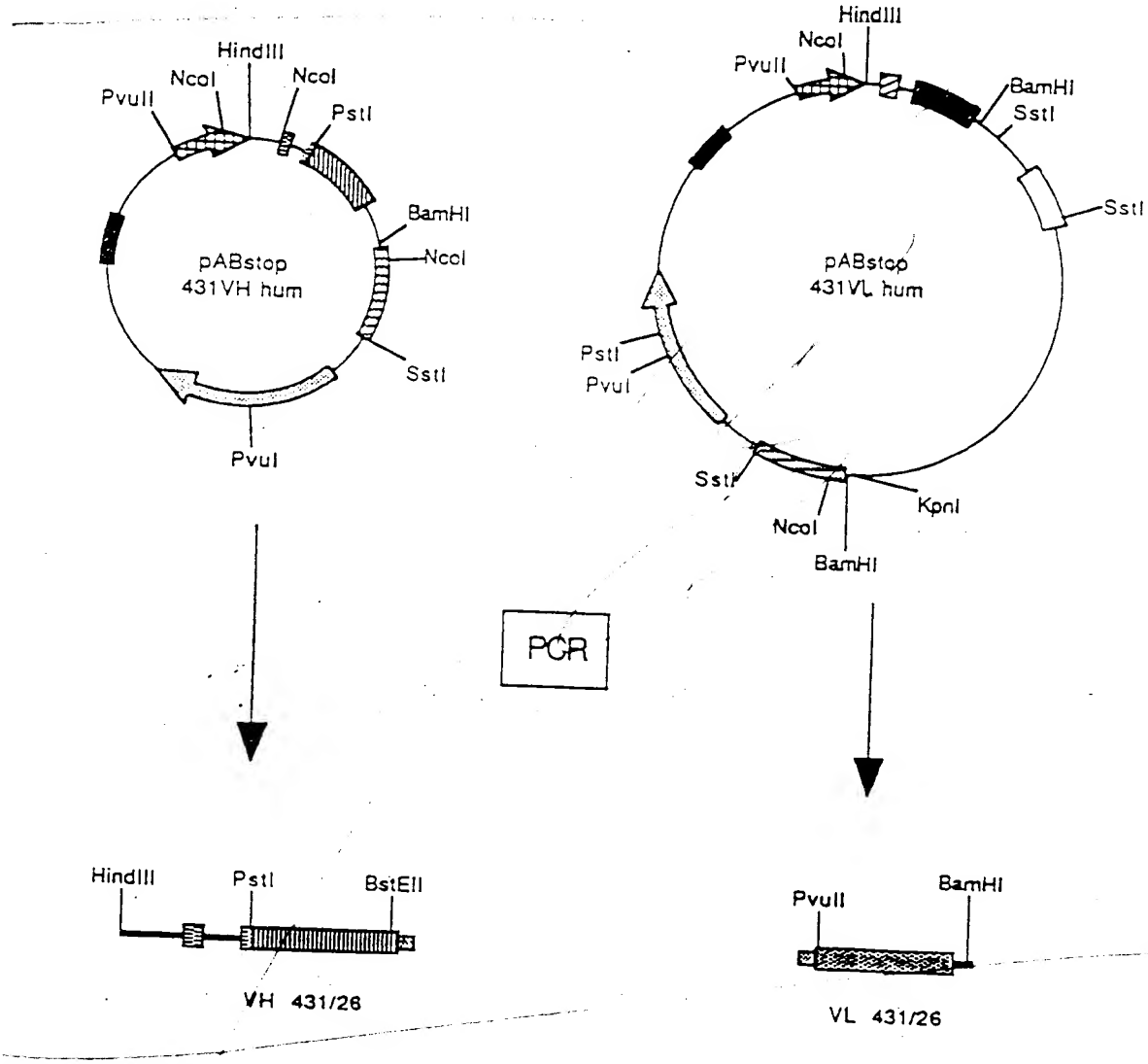
The following examples now describe the synthesis by genetic manipulation of sFv-β-Gluc fusion proteins, and the demonstration of the ability to function.

The starting material comprised the plasmids pABstop 431/26 hum V_H and pABstop 431/26 hum VH_L. These plasmids contain the humanized version of the V_H gene and V_L gene of anti-CEA MAb BW 431/26 (Güssow and Seemann, 1991, Meth. Enzymology, 203, 99-121). Further starting material comprised the plasmid pABstop 431/26 V_H-huβ-Gluc 1H (EP-A2-0 501 215) which contains a V_H exon, including the V_H-intrinsic signal sequence, followed by a CH1 exon, by the hinge exon of a human IgG3 C gene and the complete cDNA of human β-glucuronidase.

Example 1:

Amplification of the V_H and V_L genes of MAb hum 431/26

The oligonucleotides pAB-Back and linker-anti are used to amplify the V_H gene including the signal sequence intrinsic to the V_H gene from pABstop 431V_H hum (V_H 431/26) (Güssow and Seemann, 1991, Meth. Enzymology, 203, 99-121). The oligonucleotides linker-sense and $V_{L(Mut)}$-For are used to amplify the V_L gene from pABstop 431V_L hum (V_L 431/26).



Example 2:

Joining of the V_H 431/26 and V_L 431/26 gene fragments

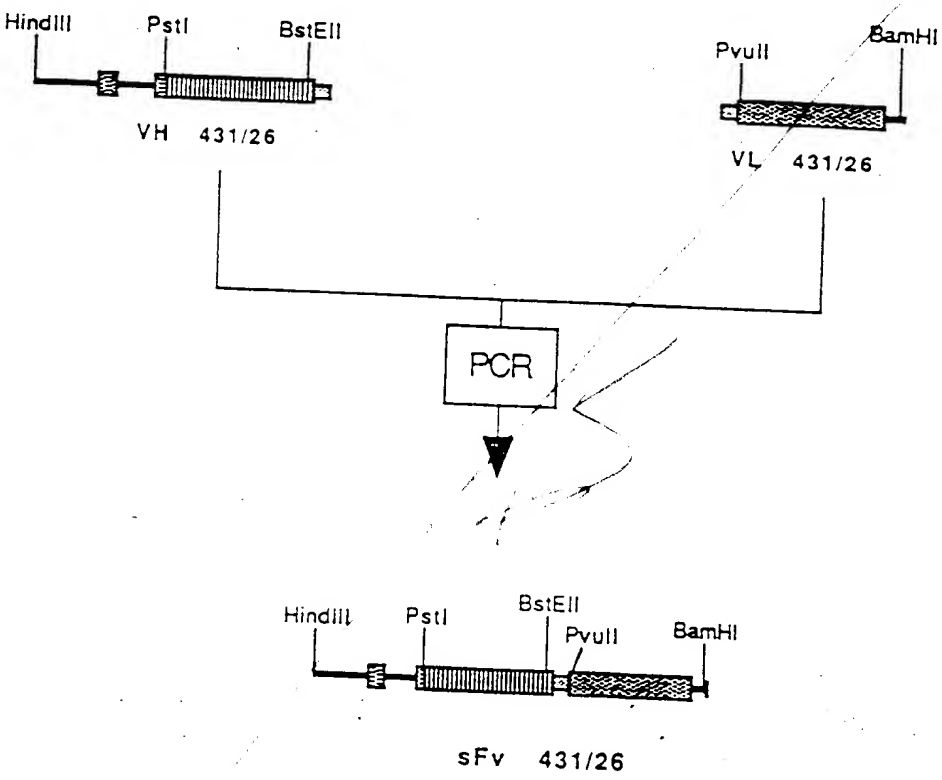
The oligonucleotides linker-anti and linker-sense are partially complementary with one another and encode a polypeptide linker which is intended to link the V_H domain and V_L domain to give an sFv fragment. In order to fuse the amplified V_H fragments with the V_L fragments, they are purified and employed in a 10-cycle reaction as follows:

H_2O:	37.5 µl
dNTPs (2.5 mM):	5.0 µl
PCR buffer (10x):	5.0 µl
Taq polymerase (Perkin-Elmer Corp., Emmeryville, CA) (2.5 U/µl):	0.5 µl
0.5 µg/µl DNA of the V_L frag.:	1.0 µl
0.5 µg/µl DNA of the V_H frag.:	1.0 µl

PCR buffer (10x): 100 mM tris, pH 8.3, 500 mM KCl, 15 mM MgCl2, 0.1% (w/v) gelatin.

The surface of the reaction mixture is sealed with paraffin, and subsequently the 10-cycle reaction is carried out in a PCR apparatus programmed for 94°C, 1 min; 55°C, 1 min; 72°C, 2 min. 2.5 pmol of the flanking primer pAB-Back and $V_{L(Mut)}$-For are added, and a further 20 cycles are carried out. The resulting PCR fragment is composed of the V_H gene which is linked to the V_L gene via a linker. The signal sequence intrinsic to the V_H gene is also present in front of the V_H gene.

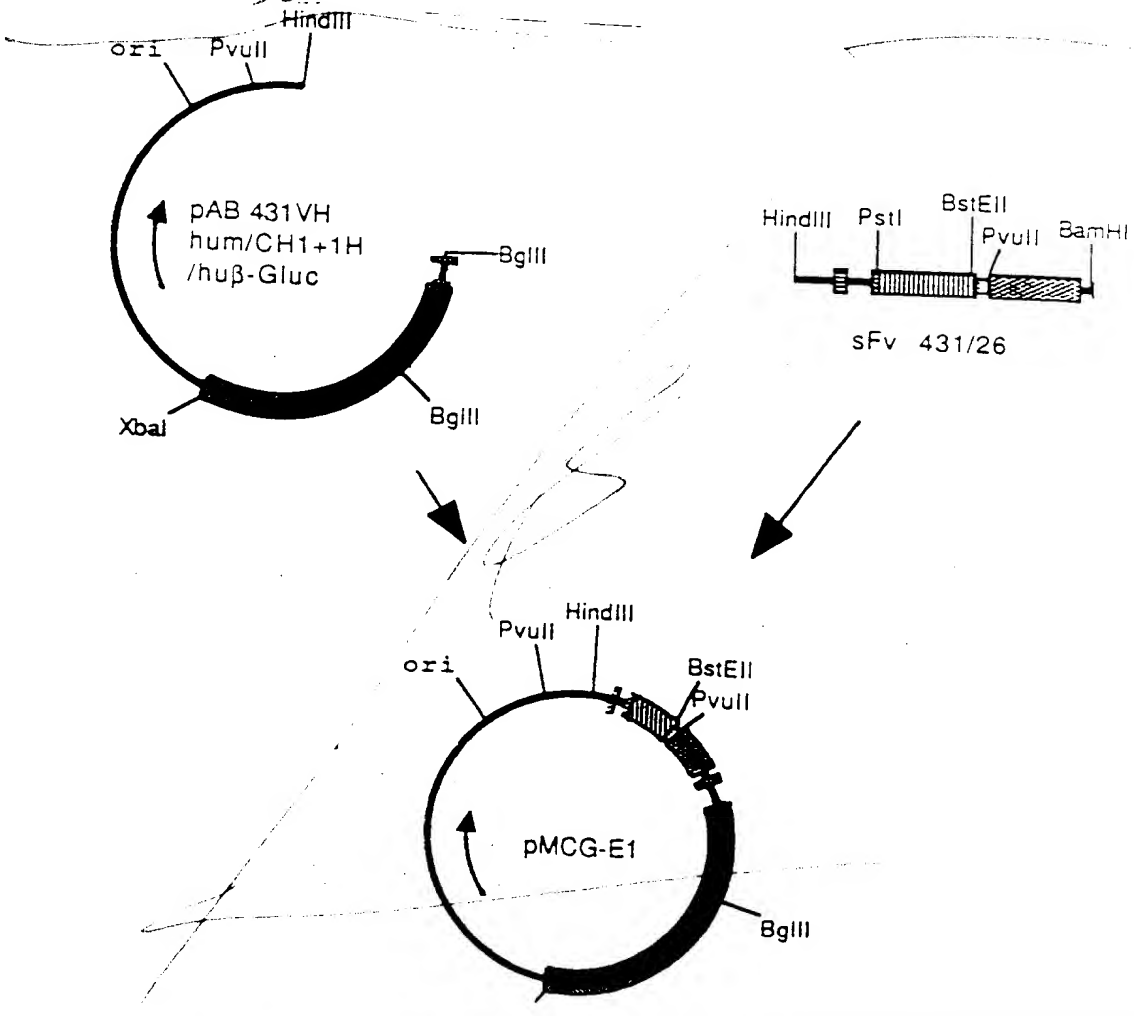
The oligonucleotide $V_{L(Mut)}$-For also results in the last nucleotide base of the V_L gene, a C, being replaced by a G. This PCR fragment codes for a humanized single-chain Fv (sFv 431/26).



Example 3:

Cloning of the sFv 431/26 fragment into the expression vector which contains the huβ-glucuronidase gene.

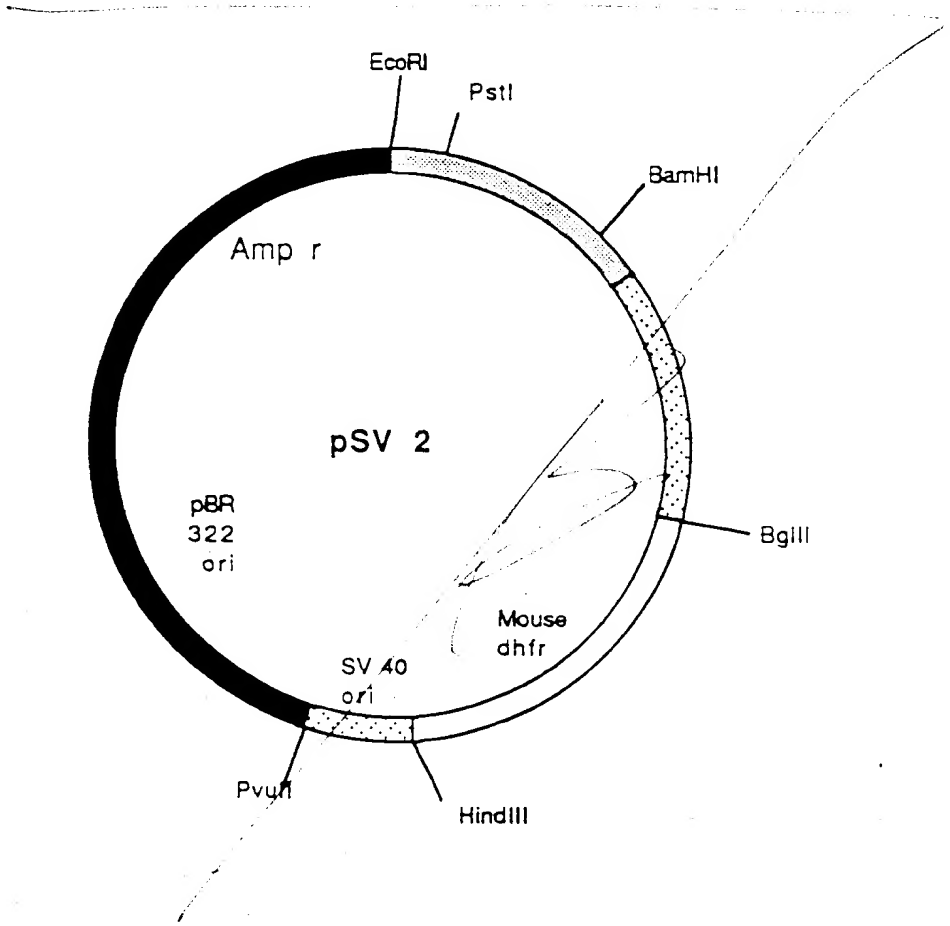
The sFv fragment from (2) is cut with HindIII and BamHI and ligated into the vector pAB 431V_H hum/CH1 + 1h/β-Glc which has been completely cleaved with HindIII and partially cleaved with BglII. The vector pABstop 431/26$V_H$huβ-Gluc1H contains a V_H exon, including the V_H-intrinsic signal sequence, followed by a CH1 exon, by the hinge exon of a human IgG3 C gene and by the complete cDNA of human β-glucuronidase. The plasmid clone pMCG-E1 which contains the humanized sFv 431/26, a hinge exon and the gene for human β-glucuronidase is isolated (pMCG-E1).

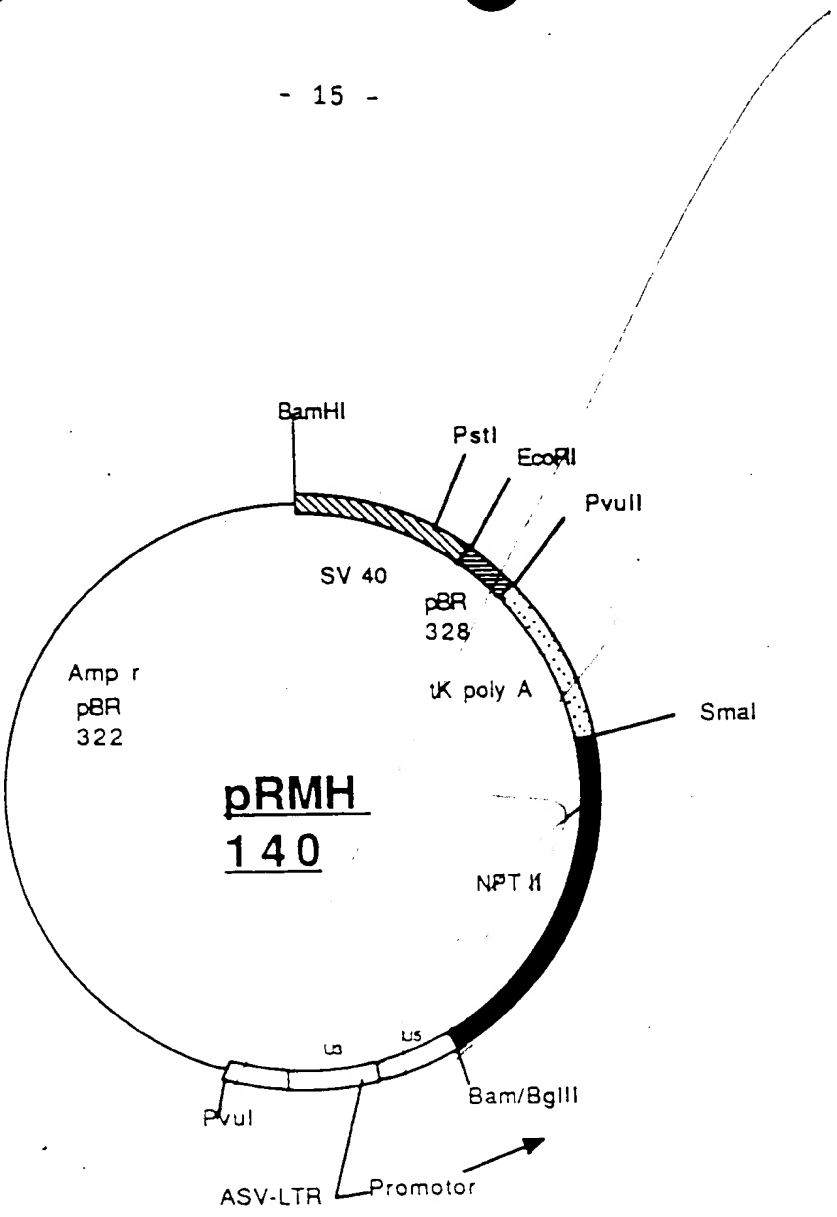


Example 4:

Expression of the sFv-huβ-Gluc fusion protein in BHK cells.

The clone pMCG-E1 is transfected with the plasmid pRMH 140 which harbors a neomycin-resistance gene and with the plasmid pSV2 which harbors a methotrexateresistance gene into BHK cells. The BHK cells subsequently express a fusion protein which has both the antigen-binding properties of MAb BW 431/26hum and the enzymatic activity of human β-glucuronidase.




BamHI
PstI
EcoRI
PvuII
SV 40
pBR
328
Amp r
pBR
322
tK poly A
SmaI
pRMH
140
NPT II
Bam/BglII
PvuI
ASV-LTR
Promotor

Example 5:

Demonstration of the antigen-binding properties and of the enzymatic activity of the sFv-huβ-Gluc fusion protein.

The ability of the sFv-huβ-Gluc fusion protein to bind specifically to the CEA epitope defined by 431/26 and simultaneously to exert the enzymatic activity of human β-glucuronidase was shown in a specificity enzyme activity test (EP-A2-0 501 215). The test determines the liberation of 4-methylumbelliferone from 4-methylumbelliferyl β-glucuronide by the β-glucuronidase portion of the fusion protein after the fusion protein has been bound via the sFv portion to an antigen. The measured fluorescence values are reported as relative fluorescence units (FU). The test shows a significant liberation of methyl-umbelliferone by the fusion protein in the plates coated with CEA. By contrast, the fusion protein does not liberate any methylumbelliferone in control plates coated with PEM (polymorphic epithelial mucin).

Example 6:

TSK 3000 gel chromatography

200 ng of the sFv-huβ-Gluc fusion protein which had been purified by anti-idiotype affinity chromatography in 25 µl were chromatographed on a TSK gel G 3000 SW XL column (TOSO HAAS Order No. 3.5Wx N3211, 7.8 mm x 300 mm) in a suitable mobile phase (PBS, pH 7.2, containing 5 g/l maltose and 4.2 g/l arginine) at a flow rate of 0.5 ml/ min. The Merck Hitachi HPLC system (L-4000 UV detector, L-6210 intelligent pump, D-2500 Chromato-integrator) was operated under ≈ 20 bar, the optical density of the eluate was determined at 280 nm, and an LKB 2111 Multisac fraction collector was used to collect 0.5 ml fractions which were subsequently analysed in a specificity enzyme activity test (SEAT) (EP 0 501 215 A2, Example J). The result of this experiment is shown in Fig. 1. It is clearly evident that the position of the peak detectable by measurement of the optical density at 280 nm coincides with the peak which determines the specificity and enzyme activity (SEAT) of the eluate. Based on the positions of the molecular weights of standard proteins which are indicated by arrows, it can be concluded that the functionally active sFv-huβ-Gluc fusion protein has an approximate molecular weight of ≈ 200 kDa under native conditions.

Example 7:

Workup of organs/tumors for determination of the fusion protein

The following sequential steps were carried out:

- nude mice (CD1) which have a subcutaneous tumor and have been treated with fusion protein or antibody-enzyme conjugate undergo retroorbital exsanguination and are then sacrificed
- the blood is immediately placed in an Eppendorf tube which already contains 10 µl of Liquemin 25000 (from Hoffman-LaRoche AG)
- centrifugation is then carried out in a centrifuge (Megafuge 1.0, from Heraeus) at 2500 rpm for 10 min
- the plasma is then obtained and frozen until tested
- the organs or the tumor are removed and weighed
- they are then completely homogenized with 2 ml of 1% BSA in PBS, pH 7.2
- the tumor homogenates are adjusted to pH 4.2 with 0.1 N HCl (the sample must not be overtitrated because β-glucuronidase is inactivated at pH < 3.8)
- all the homogenates are centrifuged at 16000 g for 30 min
- the clear supernatant is removed
- the tumor supernatants are neutralized with 0.1 N NaOH
- the supernatants and the plasma can now be quantified in immunological tests.

Example 8:

Triple determinant test

The tests are carried out as follows:

- 75 µl of a mouse anti-huβ-Gluc antibody (MAb 2118/157 Behringwerke) diluted to 2 µg/ml in PBS, pH 7.2, are placed in each well of a microtiter plate (polystyrene U-shape, type B, from Nunc, Order No. 4-60445)
- the microtiter plates are covered and incubated at R.T. overnight
- the microtiter plates are subsequently washed 3x with 250 µl of 0.05 M tris-citrate buffer, pH 7.4, per well
- these microtiter plates coated in this way are incubated with 250 µl of blocking solution (1% casein in PBS, pH 7.2) in each well at R.T. for 30' (blocking of non-specific binding sites) (coated microtiter plates which are not required are dried at R.T. for 24 hours and then sealed together with drying cartridges in coated aluminum bags for long-term storage)
- during the blocking, in an untreated 96-well U-shaped microtiter plate (polystyrene, from Renner, Order No. 12058), 10 samples + 2 positive controls + 1 negative control are diluted 1:2 in 1% casein in PBS, pH 7.2, in 8 stages (starting from 150 µl of sample, 75 µl of sample are pipetted into 75 µl of casein solution etc.)
- the blocking solution is aspirated out of the microtiter plate coated with anti-huβ-Gluc antibodies, and 50 µl of the diluted samples are transferred per well from the dilution plate to the test plate and incubated at R.T. for 30 min

- during the sample incubation, the ABC-AP reagent (from Vectastain, Order No. AK-5000) is made up: thoroughly mix 2 drops of reagent A (Avidin DH) in 10 ml of 1% casein in PBS, pH 7.2, add 2 drops of reagent B (biotinylated alkaline phosphatase) add mix thoroughly. (The ABC-AP solution must be made up at least 30' before use.)
- the test plate is washed 3 times with ELISA washing buffer (Behringwerke, Order No. OSEW 96)
- 50 µl of biotin-labeled detecting antibody mixture (1 + 1 mixture of mouse anti 431/26 antibody (MAb 2064/353, Behringwerke) and mouse anti-CEA antibody (MAb 250/183, Behringwerke) in a concentration of 5 µg/ml diluted in 1% casein in PBS, pH 7.2, final concentration of each antibody of 2.5 µg/ml) are placed in each well
- the test plate is washed 3 times with ELISA washing buffer
- 50 µl of the prepared ABC-AP solution are placed in each well and incubated at R.T. for 30 min
- during the ABC-AP incubation, the substrate is made up (fresh substrate for each test: 1 mM 4-methylumbelliferyl phosphate, Order No. M-8883, from Sigma, in 0.5 M tris + 0.01% $MgCl_2$, pH 9.6)
- the test plate is washed 7 times with ELISA washing buffer
- 50 µl of substrate are loaded into each well, and the test plate is covered and incubated at 37°C for 2 h
- 150 µl of stop solution (0.2 M glycine + 0.2% SDS, pH 11.7) are subsequently added to each well
- the fluorometric evaluation is carried out in a Fluoroscan II (ICN Biomedicals, Cat.No. 78-611-00) with an excitation wavelength of 355 nm and an emission wavelength of 460 nm

- the unknown concentration of fusion protein in the sample is determined on the basis of the fluorescence values for the positive control included in the identical experiment (dilution series with purified sFv-huβ-Gluc mixed with CEA 5 μg/ml as calibration plot).

Example 9:

Expression of the sFv-huβ-Gluc fusion protein in yeast.

The single-chain Fv (sFv) from Example 2 is amplified with the oligos 2577 and 2561 (Table 7) and cloned into the vector pUC19 which has been digested with XbaI/HindIII (Fig. 7).

The human β-glucuronidase gene is amplified with the oligos 2562 and 2540 (Table 8) from the plasmid pAB 431/26 $V_H$hum/CH1 + 1H/β-Gluc (Example 3) and ligated into the plasmid sFv 431/26 in pUC19 (Fig. 7) cut with BglII/HindIII (Fig. 8).

A KpnI/NcoI fragment is amplified with the oligos 2587 and 2627 (Table 9) from the sFv 431/26 and cloned into the yeast expression vector pIXY digested with KpnI/NcoI (Fig. 14).

The BstEII/HindIII fragment from the plasmid sFv 431/26 huβ-Gluc in pUC19 (Fig. 8) is ligated into the vector pIXY 120 which harbors the V_H gene, the linker and a part of the V_L gene (V_H/link/V_K part. in pIXY 120) and has been digested with BstEII/partially with HindIII (Fig. 15).

The resulting plasmid sFv 431/26 huβ-Gluc in pIXY 120 is transformed into Saccharomyces cerevisiae and the fusion protein is expressed.

Example 10:

Expression of the sFv-E.coli-β-glucuronidase fusion protein in yeast.

The E.coli glucuronidase gene is amplified from pRAJ 275 (Jefferson et al. Proc. Natl. Acad. Sci. USA, 83: 8447-8451, 1986) with the oligos 2638 and 2639 (~~Table 10~~) and ligated into sFv 431/26 in pUC19 (Example 9, Fig.) cut with BglII/HindIII (Fig.).

A BstEII/HindIII fragment from sFv 431/26 E.coli β-Gluc in pUC19 is cloned into the vector V_H/link/V_K part in pIXY 120 (Example 9, Fig.) which has been partially digested with BstEII/HindIII (Fig.).

The plasmid sFv 431/26 E.coli β-Gluc in pIXY 120 is transformed into Saccharomyces cerevisiae and the fusion protein is expressed.

Example 11:

Expression of the sFv-β-lactamase fusion protein in yeast.

The single-chain Fv (sFv) from Example 2 is amplified with the oligos 2587 and 2669 ~~(Table 11)~~ and ligated into the pUC19 vector digested with KpnI/HindIII (Fig. ~~8~~).

The β-lactamase II gene (Hussain et al., J. Bacteriol. 164: 223-229, 1985) is amplified with the oligos 2673 and 2674 ~~(Table 11)~~ from the complete DNA of Bacillus cereus and ligated into the pUC19 vector digested with EcoRI/HindIII (Fig. ~~9~~). A BclI/HindIII fragment of the β-lactamase gene is ligated into sFv 431/26 in pUC19 which has been cut with BglII/HindIII (Fig. ~~10~~).

The KpnI/HindIII sFv-β-lactamase fragment is ligated into pIXY 120 which has been digested with KpnI/partially with HindIII (Fig. ~~11~~). The plasmid is transformed into Saccharomyces cerevisiae, and a fusion protein which has both the antigen-binding properties of MAb 431/26 and the enzymatic activity of Bacillus cereus β-lactamase is expressed.

Table 1:

```
CCAAGCTTAT GAATATGCAA ATCCTGCTCA TGAATATGCA AATCCTCTGA                    50

ATCTACATGG TAAATATAGG TTTGTCTATA CCACAAACAG AAAAACATGA                   100

GATCACAGTT CTCTCTACAG TTACTGAGCA CACAGGACCT CACC ATG GGA TGG             153
                                                 Met Gly Trp

AGC TGT ATC ATC CTC TTC TTG GTA GCA ACA GCT ACA GGTAAGGGGC               199
Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr
                -10
TCACAGTAGC AGGCTTGAGG TCTGGACATA TATATGGGTG ACAATGACAT                   249

CCACTTTGCC TTTCTCTCCA CA GGT GTC CAC TCC CAG GTC CAA CTG CAG             298
                       Gly Val His Ser Gln Val Gln Leu Gln
                                    1
GAG AGC GGT CCA GGT CTT GTG AGA CCT AGC CAG ACC CTG AGC CTG              343
Glu Ser Gly Pro Gly Leu Val Arg Pro Ser Gln Thr Leu Ser Leu
            10                                          20
ACC TGC ACC GTG TCT GGC TTC ACC ATC AGC AGT GGT TAT AGC TGG              388
Thr Cys Thr Val Ser Gly Phe Thr Ile Ser Ser Gly Tyr Ser Trp
                        30
CAC TGG GTG AGA CAG CCA CCT GGA CGA GGT CTT GAG TGG ATT GGA              433
His Trp Val Arg Gln Pro Pro Gly Arg Gly Leu Glu Trp Ile Gly
            40                                          50
TAC ATA CAG TAC AGT GGT ATC ACT AAC TAC AAC CCC TCT CTC AAA              478
Tyr Ile Gln Tyr Ser Gly Ile Thr Asn Tyr Asn Pro Ser Leu Lys
                        60
AGT AGA GTG ACA ATG CTG GTA GAC ACC AGC AAG AAC CAG TTC AGC              523
Ser Arg Val Thr Met Leu Val Asp Thr Ser Lys Asn Gln Phe Ser
            70                                          80
CTG AGA CTC AGC AGC GTG ACA GCC GCC GAC ACC GCG GTC TAT TAT              568
Leu Arg Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val Tyr Tyr
                        90
TGT GCA AGA GAA GAC TAT GAT TAC CAC TGG TAC TTC GAT GTC TGG              613
Cys Ala Arg Glu Asp Tyr Asp Tyr His Trp Tyr Phe Asp Val Trp
            100                                         110
GGC CAA GGG ACC ACG GTC ACC GTC TCC TCA GGA GGC GGT GGA TCG              658
Gly Gln Gly Thr Thr Val Thr Val Ser Ser Gly Gly Gly Gly Ser
                        120
GGC GGT GGT GGG TCG GGT GGC GGC GGA TCT GAC ATC CAG CTG ACC              703
Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp Ile Gln Leu Thr
            130                                         140
CAG AGC CCA AGC AGC CTG AGC GCC AGC GTG GGT GAC AGA GTG ACC              748
Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly Asp Arg Val Thr
                        150
ATC ACC TGT AGT ACC AGC TCG AGT GTA AGT TAC ATG CAC TGG TAC              793
Ile Thr Cys Ser Thr Ser Ser Ser Val Ser Tyr Met His Trp Tyr
            160                                         170
CAG CAG AAG CCA GGT AAG GCT CCA AAG CTG CTG ATC TAC AGC ACA              838
Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr Ser Thr
                        180
```

Table 1 (Continuation):

```
TCC AAC CTG GCT TCT GGT GTG CCA AGC AGA TTC AGC GGT AGC GGT          883
Ser Asn Leu Ala Ser Gly Val Pro Ser Arg Phe Ser Gly Ser Gly
                190                                 200
AGC GGT ACC GAC TTC ACC TTC ACC ATC AGC AGC CTC CAG CCA GAG          928
Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Leu Gln Pro Glu
                            210
GAC ATC GCC ACC TAC TAC TGC CAT CAG TGG AGT AGT TAT CCC ACG          973
Asp Ile Ala Thr Tyr Tyr Cys His Gln Trp Ser Ser Tyr Pro Thr
            220                                 230
TTC GGC CAA GGG ACC AAG CTG GAG ATC AAA GGTGAGTAGA ATTTAAACTT       1023
Phe Gly Gln Gly Thr Lys Leu Glu Ile Lys
                        240
TGCTTCCTCA GTTGGATCTG AGTAACTCCC AATCTTCTCT CTGCA GAG CTC AAA       1077
                                            Glu Leu Lys
ACC CCA CTT GGT GAC ACA ACT CAC ACA TGC CCA CGG TGC CCA             1119
Thr Pro Leu Gly Asp Thr Thr His Thr Cys Pro Arg Cys Pro
                    250
GGTAAGCCAG CCCAGGACTC GCCCTCCAGC TCAAGGCGGG ACAAGAGCCC              1169

TAGAGTGGCC TGAGTCCAGG GACAGGCCCC AGCAGGGTGC TGACGCATCC              1219

ACCTCCATCC CAGATCCCCG TAACTCCCAA TCTTCTCTCT GCA GCG GCG GCG         1271
                                                Ala Ala Ala
                                                        260
GCG GTG CAG GGC GGG ATG CTG TAC CCC CAG GAG AGC CCG TCG CGG         1316
Ala Val Gln Gly Gly Met Leu Tyr Pro Gln Glu Ser Pro Ser Arg
                                270
GAG TGC AAG GAG CTG GAC GGC CTC TGG AGC TTC CGC GCC GAC TTC         1361
Glu Cys Lys Glu Leu Asp Gly Leu Trp Ser Phe Arg Ala Asp Phe
            280                                 290
TCT GAC AAC CGA CGC CGG GGC TTC GAG GAG CAG TGG TAC CGG CGG         1406
Ser Asp Asn Arg Arg Arg Gly Phe Glu Glu Gln Trp Tyr Arg Arg
                            300
CCG CTG TGG GAG TCA GGC CCC ACC GTG GAC ATG CCA GTT CCC TCC         1451
Pro Leu Trp Glu Ser Gly Pro Thr Val Asp Met Pro Val Pro Ser
                310                                 320
AGC TTC AAT GAC ATC AGC CAG GAC TGG CGT CTG CGG CAT TTT GTC         1496
Ser Phe Asn Asp Ile Ser Gln Asp Trp Arg Leu Arg His Phe Val
                            330
GGC TGG GTG TGG TAC GAA CGG GAG GTG ATC CTG CCG GAG CGA TGG         1541
Gly Trp Val Trp Tyr Glu Arg Glu Val Ile Leu Pro Glu Arg Trp
            340                                 350
ACC CAG GAC CTG CGC ACA AGA GTG GTG CTG AGG ATT GGC AGT GCC         1586
Thr Gln Asp Leu Arg Thr Arg Val Val Leu Arg Ile Gly Ser Ala
                            360
CAT TCC TAT GCC ATC GTG TGG GTG AAT GGG GTC GAC ACG CTA GAG         1631
His Ser Tyr Ala Ile Val Trp Val Asn Gly Val Asp Thr Leu Glu
            370                                 380
CAT GAG GGG GGC TAC CTC CCC TTC GAG GCC GAC ATC AGC AAC CTG         1676
His Glu Gly Gly Tyr Leu Pro Phe Glu Ala Asp Ile Ser Asn Leu
                            390
GTC CAG GTG GGG CCC CTG CCC TCC CGG CTC CGA ATC ACT ATC GCC         1721
Val Gln Val Gly Pro Leu Pro Ser Arg Leu Arg Ile Thr Ile Ala
            400                                 410
```

Table 1 (Continuation):

```
ATC AAC AAC ACA CTC ACC CCC ACC ACC CTG CCA CCA GGG ACC ATC      1766
Ile Asn Asn Thr Leu Thr Pro Thr Thr Leu Pro Pro Gly Thr Ile
                                    420
CAA TAC CTG ACT GAC ACC TCC AAG TAT CCC AAG GGT TAC TTT GTC      1811
Gln Tyr Leu Thr Asp Thr Ser Lys Tyr Pro Lys Gly Tyr Phe Val
            430                                         440
CAG AAC ACA TAT TTT GAC TTT TTC AAC TAC GCT GGA CTG CAG CGG      1856
Gln Asn Thr Tyr Phe Asp Phe Phe Asn Tyr Ala Gly Leu Gln Arg
                                    450
TCT GTA CTT CTG TAC ACG ACA CCC ACC ACC TAC ATC GAT GAC ATC      1901
Ser Val Leu Leu Tyr Thr Thr Pro Thr Thr Tyr Ile Asp Asp Ile
            460                                         470
ACC GTC ACC ACC AGC GTG GAG CAA GAC AGT GGG CTG GTG AAT TAC      1946
Thr Val Thr Thr Ser Val Glu Gln Asp Ser Gly Leu Val Asn Tyr
                                    480
CAG ATC TCT GTC AAG GGC AGT AAC CTG TTC AAG TTG GAA GTG CGT      1991
Gln Ile Ser Val Lys Gly Ser Asn Leu Phe Lys Leu Glu Val Arg
            490                                         500
CTT TTG GAT GCA GAA AAC AAA GTC GTG GCG AAT GGG ACT GGG ACC      2036
Leu Leu Asp Ala Glu Asn Lys Val Val Ala Asn Gly Thr Gly Thr
                                    510
CAG GGC CAA CTT AAG GTG CCA GGT GTC AGC CTC TGG TGG CCG TAC      2081
Gln Gly Gln Leu Lys Val Pro Gly Val Ser Leu Trp Trp Pro Tyr
            520                                         530
CTG ATG CAC GAA CGC CCT GCC TAT CTG TAT TCA TTG GAG GTG CAG      2126
Leu Met His Glu Arg Pro Ala Tyr Leu Tyr Ser Leu Glu Val Gln
                                    540
CTG ACT GCA CAG ACG TCA CTG GGG CCT GTG TCT GAC TTC TAC ACA      2171
Leu Thr Ala Gln Thr Ser Leu Gly Pro Val Ser Asp Phe Tyr Thr
            550                                         560
CTC CCT GTG GGG ATC CGC ACT GTG GCT GTC ACC AAG AGC CAG TTC      2216
Leu Pro Val Gly Ile Arg Thr Val Ala Val Thr Lys Ser Gln Phe
                                    570
CTC ATC AAT GGG AAA CCT TTC TAT TTC CAC GGT GTC AAC AAG CAT      2261
Leu Ile Asn Gly Lys Pro Phe Tyr Phe His Gly Val Asn Lys His
            580                                         590
GAG GAT GCG GAC ATC CGA GGG AAG GGC TTC GAC TGG CCG CTG CTG      2306
Glu Asp Ala Asp Ile Arg Gly Lys Gly Phe Asp Trp Pro Leu Leu
                                    600
GTG AAG GAC TTC AAC CTG CTT CGC TGG CTT GGT GCC AAC GCT TTC      2351
Val Lys Asp Phe Asn Leu Leu Arg Trp Leu Gly Ala Asn Ala Phe
            610                                         620
CGT ACC AGC CAC TAC CCC TAT GCA GAG GAA GTG ATG CAG ATG TGT      2396
Arg Thr Ser His Tyr Pro Tyr Ala Glu Glu Val Met Gln Met Cys
                                    630
GAC CGC TAT GGG ATT GTG GTC ATC GAT GAG TGT CCC GGC GTG GGC      2441
Asp Arg Tyr Gly Ile Val Val Ile Asp Glu Cys Pro Gly Val Gly
            640                                         650
CTG GCG CTG CCG CAG TTC TTC AAC AAC GTT TCT CTG CAT CAC CAC      2486
Leu Ala Leu Pro Gln Phe Phe Asn Asn Val Ser Leu His His His
                                    660
ATG CAG GTG ATG GAA GAA GTG GTG CGT AGG GAC AAG AAC CAC CCC      2531
Met Gln Val Met Glu Glu Val Val Arg Arg Asp Lys Asn His Pro
            670                                         680
```

Table 1 (Continuation):

```
GCG GTC GTG ATG TGG TCT GTG GCC AAC GAG CCT GCG TCC CAC CTA    2576
Ala Val Val Met Trp Ser Val Ala Asn Glu Pro Ala Ser His Leu
                                    690
GAA TCT GCT GGC TAC TAC TTG AAG ATG GTG ATC GCT CAC ACC AAA    2621
Glu Ser Ala Gly Tyr Tyr Leu Lys Met Val Ile Ala His Thr Lys
                700                                 710
TCC TTG GAC CCC TCC CGG CCT GTG ACC TTT GTG AGC AAC TCT AAC    2666
Ser Leu Asp Pro Ser Arg Pro Val Thr Phe Val Ser Asn Ser Asn
                                    720
TAT GCA GCA GAC AAG GGG GCT CCG TAT GTG GAT GTG ATC TGT TTG    2711
Tyr Ala Ala Asp Lys Gly Ala Pro Tyr Val Asp Val Ile Cys Leu
                730                                 740
AAC AGC TAC TAC TCT TGG TAT CAC GAC TAC GGG CAC CTG GAG TTG    2756
Asn Ser Tyr Tyr Ser Trp Tyr His Asp Tyr Gly His Leu Glu Leu
                                    750
ATT CAG CTG CAG CTG GCC ACC CAG TTT GAG AAC TGG TAT AAG AAG    2801
Ile Gln Leu Gln Leu Ala Thr Gln Phe Glu Asn Trp Tyr Lys Lys
                760                                 770
TAT CAG AAG CCC ATT ATT CAG AGC GAG TAT GGA GCA GAA ACG ATT    2846
Tyr Gln Lys Pro Ile Ile Gln Ser Glu Tyr Gly Ala Glu Thr Ile
                                    780
GCA GGG TTT CAC CAG GAT CCA CCT CTG ATG TTC ACT GAA GAG TAC    2891
Ala Gly Phe His Gln Asp Pro Pro Leu Met Phe Thr Glu Glu Tyr
                790                                 800
CAG AAA AGT CTG CTA GAG CAG TAC CAT CTG GGT CTG GAT CAA AAA    2936
Gln Lys Ser Leu Leu Glu Gln Tyr His Leu Gly Leu Asp Gln Lys
                                    810
CGC AGA AAA TAT GTG GTT GGA GAG CTC ATT TGG AAT TTT GCC GAT    2981
Arg Arg Lys Tyr Val Val Gly Glu Leu Ile Trp Asn Phe Ala Asp
                820                                 830
TTC ATG ACT GAA CAG TCA CCG ACG AGA GTG CTG GGG ATT AAA AAG    3026
Phe Met Thr Glu Gln Ser Pro Thr Arg Val Leu Gly Asn Lys Lys
                                    840
GGG ATC TTC ACT CGG CAG AGA CAA CCA AAA AGT GCA GCG TTC CTT    3071
Gly Ile Phe Thr Arg Gln Arg Gln Pro Lys Ser Ala Ala Phe Leu
                850                                 860
TTG CGA GAG AGA TAC TGG AAG ATT GCC AAT GAA ACC AGG TAT CCC    3116
Leu Arg Glu Arg Tyr Trp Lys Ile Ala Asn Glu Thr Arg Tyr Pro
                                    870
CAC TCA GTA GCC AAG TCA CAA TGT TTG GAA AAC AGC CCG TTT ACT    3161
His Ser Val Ala Lys Ser Gln Cys Leu Glu Asn Ser Pro Phe Thr
                880                                 890
TGA GCAAGACTGA TACCACCTGC GTGTCCCTTC CTCCCCGAGT CAGGGCGACT    3214
...

TCCACAGCAG CAGAACAAGT GCCTCCTGGA CTGTTCACGG CAGACCAGAA        3264

CGTTTCTGGC CTGGGTTTTG TGGTCATCTA TTCTAGCAGG GAACACTAAA        3314
```

Table 2:

pAB-Back:

5′
ACC AGA AGC TTA TGA ATA TGC AAA TC 3′

Linker-Anti:

5′
GCC ACC CGA CCC ACC ACC GCC CGA TCC ACC GCC TCC TGA

GGA GAC GGT GAC CGT GGT C 3′

Table 3:

Linker-Sense:

5′
GGT GGA TCG GGC GGT GGT GGG TCG GGT GGC GGC GGA TCT

GAC ATC CAG CTG ACC CAG AGC 3′

VL(Mut)-For:

5′
TGC AGG ATC CAA CTG AGG AAG CAA AGT TTA AAT TCT ACT

CAC CTT TGA TC 3′

Table 1

Pharmacokinetics of sFv-hu β Gluc fusion protein in CD1 nu/nu mice carrying MzSto1

ng of sFv-huβGluc per gram of tissue or ml of plasma measured in the triple determinant test

Tissue type	Mouse 1 0.05 h	Mouse 2 3 h	Mouse 3 24 h	Mouse 4 48 h	Mouse 5a 120 h	Mouse 5b 120 h
Tumor	24.8	4	7.7	2.1	2.2	6.2
Spleen	15.4	4.1	<0.1	<0.1	<0.1	<0.1
Liver	40.9	10.1	0.8	0.8	0.3	<0.1
Intestine	5.2	4.4	1.1	1.2	0.6	<0.1
Kidney	44.4	7	<0.1	<0.1	<0.1	<0.1
Lung	154.8	17.3	<0.1	<0.1	<0.1	<0.1
Heart	148.3	8.2	<0.1	<0.1	<0.1	<0.1
Plasma	630.9	95	2.7	0.4	<0.1	<0.1

i.v. injection of 0.8 µg of purified fusion protein per mouse

Table 5

Analysis of the monosaccharide components in the carbohydrate content of the sFv-huß-Gluc fusion protein from BHK cells

The purified sFv-huß-Gluc fusion protein was investigated for its carbohydrate content. This revealed after hydrolysis the following individual components in the stated molar ratio (mol of carbohydrate/mol of sFv-huß-Gluc).

	Fucose	Galactosamine	N-Acetyl glucosamine	Galactose	Glucose	Mannose	N-Acetyl-neuraminic acid
sFv-huß-Gluc	4	2	30	8	1	43	4

The molar ratios of mannose, glucosamine and galactose allow conclusions to be drawn about the presence of the high-mannose type and/or hybrid type structures (besides complex type structures). Therefore mannose, galactose, acetylneuraminic acid and possibly N-acetylglucosamine occur terminally, and mannose may also be present as mannose 6-phosphate.

Methods:

Neuraminic acid was determined by the method of Hermentin and Seidat (1991) GBF Monographs Volume 15, pp. 185-188 (after hydrolysis for 30 min in the presence of 0.1 N sulfuric acid at 80 °C and subsequent neutralization with 0.4 N sodium hydroxide solution) by high-pH anion exchange chromatography with pulsed amerometric detection (HPAE-PAD).

The **monosaccaride components** were determined (after hydrolysis for 4 h in the presence of 2 N trifluoracetic acid at 100 °C and evaporation to dryness in a SpeedVac) likewise by HPAE-PAD in a motivation of the method described by Hardy et al. (1988) Analytical Biochemistry 170, pp. 54-62.

Table 6

Analysis of the monosaccharide components in the carbohydrate content of the sFv-huβGluc fusion protein from Saccharomyces cerevisiae.

	Glucosamine	Glucose	Mannose	
sFv-huβGluc (mol/mol)	6	12	150	mol/mol

Table 7:

Oligos for sFv 431/26 cloning in pUC 19

sFv for (2561)

5′ TTT TTA AGC TTA GAT CTC CAC CTT GGT C 3′

sFv back (2577)

5′ AAA AAT CTA GAA TGC AGG TCC AAC TGC AGG AGA G 3′

Table 8:

Oligos for hum.β-Gluc cloning in sFv pUC 19

Hum.β-Gluc. back oligo (2562)

5′ AAA AAA GTG ATC AAA GCG TCT GGC GGG CCA CAG GGC GGG ATC CTG TAC 3′

Hum.β-Gluc for oligo (2540)

5′ TTT TAA GCT TCA AGT AAA CGG GCT GTT 3′

Table 9:

Oligos for sFv/hum-β-Gluc cloning in pIXY120

PCR oligo VHpIXY back (2587)

5' TTT TGG TAC CTT TGG ATA AAA GAC AGG TCC AAC TGC AGG AGA G 3'

PCR oligo VKpIXY for (2627)

5' A AAA CCA TGG GAA TTC AAG CTT CGA GCT GGT ACT ACA GGT 3'

Table 10:

Oligos for E.coli β-Gluc cloning in sFv pUC 19

E. coli β-Gluc. for (2639)

5′ TTT TAA GCT TCC ATG GCG GCC GCT CAT TGT TTG
CCT CCC TGC TG 3′

E. coli β-Gluc. back (2638)

5′ AAA AAG ATC TCC GCG TCT GGC GGG CCA CAG TTA
CGT GTA GAA ACC CCA 3′

Table 11:

Oligos for sFv/β-lactamase cloning in pIXY120

PCR oligo VHpIXY back (2587)

5′ TTT TGG TAC CTT TGG ATA AAA GAC AGG TCC AAC TGC AGG
AGA G 3′

PCR oligo VKpIXY/β-lactamase for (2669)

5′ AAA AAG CTT AGA TCT CCA GCT TGG TCC C 3′

PCR oligo link/β-lactamase back (2673)

5′ AAA GAA TTC TGA TCA AAT CCT CGA GCT CAG GTT CAC
AAA AGG TAG AGA AAA CAG T 3′ linker

PCR oligo β-lactamase for (2674)

5′ TTT AAG CTT ATT TTA ATA AAT CCA ATG T 3′